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The effects of chain flexibility on the properties of vesicles formed from di-*n*-alkyl phosphates^a

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Abstract. This paper describes a study of the effects of chain flexibility and chain packing on the properties of vesicles formed from sodium di-*n*-alkyl phosphates. Three di-*n*-alkyl phosphates with a constant chain length but with different degrees of unsaturation have been synthesized: dioleoyl phosphate (DOP) and dielaidyl phosphate (DEP) having, respectively, a *cis* and a *trans* double bond at C-9 and the saturated distearyl phosphate (DSP). These surfactants form vesicles, as confirmed by transmission electron microscopy (EM). The gel to liquid-crystalline phase transition was studied using both fluorescence polarization and differential scanning microcalorimetry. According to fluorescence polarization, using *trans,trans,trans*-1,6-diphenyl-hexa-1,3,5-triene (DPH) as a probe, DSP vesicles undergo a cooperative transition from a gel to a liquid-crystalline state at 72°C. The polarization of the probe captured in DOP or DEP vesicles decreased gradually in the temperature range 0-40°C, indicating a non-cooperative phase transition. It appears that the vesicle bilayer is in a gel state below 0°C and in a liquid-crystalline state above 40°C. A temperature-dependent ³¹P NMR study failed to identify the exact phase-transition temperature. The effect of the fusogenic cation Ca²⁺ was studied qualitatively using EM. Calcium induces fusion of DEP vesicles but, within a short time, tubules are formed which are most probably anhydrous crystals of the calcium salts. For DSP vesicles the latter process is extremely fast and fused vesicles cannot be detected. In contrast, DOP vesicles fuse under the influence of calcium, but no crystallization takes place. The fused DOP vesicles are stable for more than one week in the presence of 4 mM Ca²⁺ stored at room temperature or at 60°C. Addition of EDTA to DOP vesicles leads to chelation of the calcium ions and to a transition to multilamellar vesicles.

Introduction

Essential biochemical functions are associated with the structural properties of the biological cell membrane¹. Proteins, sugars, glycolipids and cholesterol are located in the phospholipid bilayer which results in a complex supramolecular assembly. Consequently, it is difficult to study properties of cell membranes directly and many studies have been performed using phospholipid vesicles (liposomes) as model systems². In 1977, Kunitake³ showed that simple synthetic surfactant molecules also form bilayer vesicles⁴. These aggregates have many features in common with cell membranes. This discovery opened the field of membrane-mimetic chemistry⁵. The relation between surfactant structure and vesicle properties is interesting in the context of stability, phase transitions, fusion behaviour, and the potential use of vesicles formed from synthetic amphiphiles in drug targeting.

Relation between surfactant structure and vesicle properties

Although the alkyl chains in a vesicle bilayer are markedly ordered, a vesicle bilayer is a dynamic assembly. There are three important dynamic processes: (i) movement of the alkyl chains with respect to the headgroup, (ii) lateral diffusion of monomers through one bilayer leaflet and (iii) flip-flop movement of a molecule from the inner to the outer monolayer^{5,6}. The dynamics of these processes are primarily influenced by the nature of the headgroup and the length and flexibility of the alkyl chains. Recently, we studied the effect of alkyl-chain length and chain asymmetry in vesicles formed from both symmetric (equal chain lengths) and asymmetric (unequal chain lengths) di-*n*-alkyl phosphates^{7,8}. All di-*n*-alkyl phosphates with sufficiently long alkyl chains were found to form vesicles, despite large differences in chain length. In the study reported here, we have examined the influence of chain flexibility on the properties of di-*n*-alkyl phosphate vesicles. Unsaturation in the alkyl chains of lipids is known to have a dramatic influence on the phase-transition temperatures of the corresponding liposomes^{9,10,11,12}. Here, the effect of chain flexibility and double bond geometry on

^a Dedicated to Professor Upendra Pandit on the occasion of his retirement from the Department of Organic Chemistry of the University of Amsterdam.

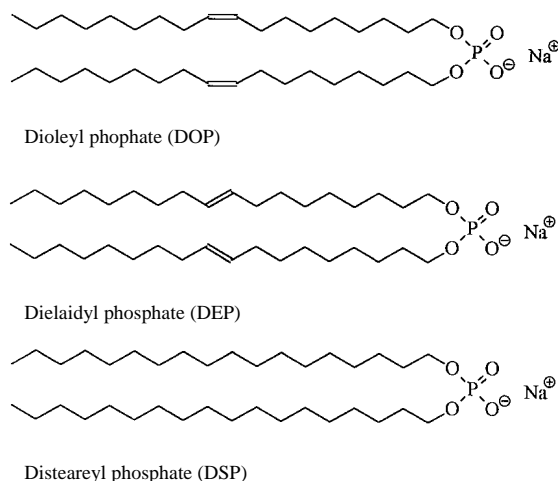


Figure 1. Structures of the sodium di- *n*-alkyl phosphates 1-3.

the chain packing were studied by comparing the properties of the vesicles formed from three di-*n*-alkyl phosphates with constant chain length (18 carbon atoms) but different degrees of unsaturation. The di-*n*-alkyl phosphates are: sodium dioleoyl phosphate (DOP, **1**) and sodium dielaidyl phosphate (DEP, **2**) having, respectively, a *cis* and a *trans* double bond at C-9, and the saturated sodium distearyl phosphate (DSP, **3**).

Experimental

Synthesis of sodium di- *n*-alkyl phosphates

Sodium dioleoyl^b phosphate and sodium dielaidyl^b phosphate were synthesized by a modification of the procedure described by Kunitake¹³. Two equivalents of octadec-9-en-1-ol were refluxed with POCl₃ in benzene for 24 h, followed by hydrolysis in an acetone/water (1/40) mixture. Dielaidyl phosphate was purified by crystallization from 96% ethanol and converted into the sodium salt with sodium ethoxide. ¹H NMR: **d** 0.88 ppm (t, CH₃); 1.27 (m, 11 CH₂ alkyl chain); 1.65 (m, CH₂CH₂-OP); 1.97 (m, CH₂-CH=CH-CH₂); 4.00 (d, CH₂-OP); 5.38 (t, CH=CH). ¹³C NMR: **d** 14.04 ppm, (CH₃); 22.6-33.5 (CH₂ alkyl chain); 67.51, 67.60 (d, CH₂-OP, J = 6.91 Hz); 130.08, 130.29 (CH=CH, *trans*). Anal. calc.: C 72.19; H 11.95; P 5.17; found: C 72.30; H 11.89; P 5.02%.

Dioleoyl phosphate¹⁴ was converted into its sodium salt and then crystallized at low temperature from a methanol/acetone mixture. ¹³C NMR spectroscopic data indicated that DOP was not contaminated with the *trans* isomer.

Distearyl phosphate (mp. 82°C)¹⁵ and the corresponding sodium salt were prepared by A. Wagenaar using the same method as was used for the shorter-chain di-*n*-alkyl phosphates¹⁶.

Preparation of vesicles

The vesicles were prepared from 5 mg of sodium di-*n*-alkyl phosphate dissolved in 50 μ l of 96% ethanol. Of this solution 40 μ l was injected into a well-stirred buffer solution (1 ml) at a temperature above the phase-transition temperature (DOP, DEP: 55°C; DSP: 80°C). The buffer was a low-salt (5 mM sodium acetate) 4-(2-hydroxyethyl)-piperazine-1-ethane sulfonic acid (HEPES) buffer, pH 7.4. The vesicles were freshly prepared for each experiment or stored (for no longer than one day) in an oven at 60°C, (75°C for DSP). The concentration of surfactant in these solutions was $6.44 \cdot 10^{-3}$ M.

Fluorescence polarization

The phase-transition behaviour of DOP, DEP and DSP vesicles was studied by fluorescence polarization. The rod-like probe molecule *trans,trans,trans*-1,6-diphenyl-hexa-1,3,5-triene (DPH) is entrapped in the bilayer. Polarized light is absorbed by the fluorescent molecules

and subsequently emitted. The polarization differences between emitted and absorbed light is characterized by the polarization *P*:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where I_{\perp} is light perpendicularly polarized and I_{\parallel} light with a polarization parallel to the absorbed light. Vesicle bilayers in the gel state typically exhibit *P* values in the range of 0.3-0.4, whereas *P* values in the range of 0-0.1 are found for bilayers in the liquid-crystalline state.

In the experiment a freshly prepared vesicle solution was diluted to yield a $4.0 \cdot 10^{-5}$ M solution. 5 μ l of a $5 \cdot 10^{-5}$ M solution of DPH in tetrahydrofuran was added to 5 ml of the vesicle solution, to give a final DPH concentration of $5 \cdot 10^{-8}$ M. Fluorescence spectra were measured using an SLM Aminco SPF-500C spectrofluorometer. The emission wavelength was 480 nm, the excitation wavelength was 360 nm and the bandpass was 5 nm. At each temperature the polarization was measured and calibrated three times.

³¹P NMR spectroscopy

The ³¹P NMR spectra were recorded on a Varian VXR 300-MHz spectrometer under carefully controlled conditions¹⁵. The samples were prepared by the ethanol-injection method and 10% of D₂O was added. Line-width measurements were initiated at the highest temperature and were performed in temperature steps of 3 or 5°C. At each temperature the sample was equilibrated over a period of 15 min. These experiments were performed by A. Wagenaar.

Electron microscopy samples

Vesicles were prepared using the standard procedure. Ca²⁺ was added by injecting 10 μ l of a 1.4 M solution of CaCl₂ into 1 ml of a stirred vesicle solution to obtain a final Ca²⁺ concentration of 4 mM. EDTA was added in a two-fold excess. Vesicle dispersions were stained on carbon-coated Formvar grids according to the two-droplet method with 1% or 0.1% (w/v) solution of uranyl acetate¹⁷. The samples were examined in a Philips EM-300 electron microscope operating at 80-kV accelerating voltage.

Differential scanning microcalorimetry

Dependences on temperature of the differential isobaric heat capacities of water and vesicular solutions were measured using a differential-scanning microcalorimeter (Microcal MC-2 microcalorimeter, Microcal, Amherst MA) fitted for enhanced sensitivity with a nanovoltmeter¹⁸. The microcalorimeter compared the heat capacities of water and vesicular solutions with the sample and reference volumes being approximately 1.2 ml. The scan rate was approximately 60°C per hour. Since microcalorimetric studies had previously shown that the ethanol-injection method may provide irreproducible results in that the scan pattern was not reversible for a single sample¹⁹, the vesicles were prepared by the hot water method, by dissolving a known weight in a known volume of water²⁰.

Results and discussion

Electron-microscopic (EM) study of di-*n*-alkyl phosphate vesicles

Convincing evidence for the formation of vesicles from the sodium di-*n*-alkyl phosphates **1-3** was obtained using electron microscopy. This technique showed that DOP, DEP and DSP all form vesicles despite large differences in chain flexibility. The DOP vesicles are spherical and vary in size from 40 to 100 nm (Figure 7a, *vide infra*); DEP vesicles are collapsed, with diameters 90-130 nm, while DSP vesicles are larger with diameters in the range of 100-600 nm. Some of the DSP vesicles are spherical, some are collapsed.

Effects of chain flexibility

A change in chain flexibility has a large influence on aggregate properties²¹. In 1-methyl-4-dodecylpyridinium

^b Oleoyl = (Z)- and elaidyl = (E)-octadec-9-enyl.

fluorescence polarization DSP vesicles

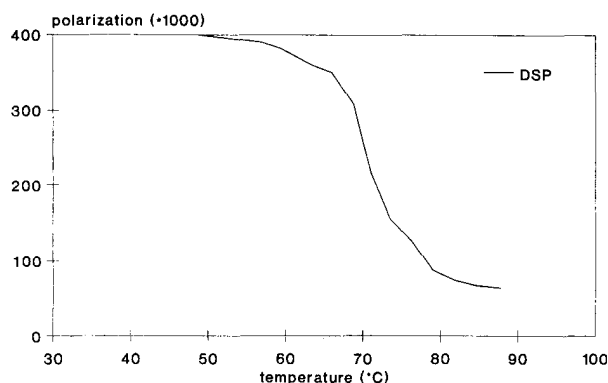


Figure 2. Plot of the fluorescence polarization (P) vs. T ($^{\circ}\text{C}$) for DSP.

iodide surfactants, the introduction of a triple bond in the middle of the chain resulted in a five-fold increase in the critical micellar concentration (CMC) ($2.45 \cdot 10^{-3}$ for the saturated and $13 \cdot 10^{-3} \text{ mol} \cdot \text{kg}^{-1}$, respectively, for the acetylene analogue)²². This increase in CMC , which reflects a decrease of micelle stability, is attributed to a negative influence of the acetylene moiety on the efficiency of alkyl chain packing. The differences in chain flexibility are also reflected in the gel to liquid-crystalline phase-transition temperatures (T_m) of phosphatidylcholine (PC) liposomes²³. Introduction of one double bond with a *cis* configuration causes a lowering of the T_m by 70°C ²⁴. The effect of a *trans* double bond is much smaller. Here we show that similar striking effects are observed for vesicles formed from di-*n*-alkyl phosphates.

Fluorescence polarization

For DSP vesicles the polarization ratio P as a function of temperature is shown in Figure 2. Starting at high temperature, there is a steep increase in polarization over a narrow temperature range and the highly cooperative phase transition is found at 72°C .

Similar plots for DEP and DOP (Figure 3) show that in these cases the phase transition is non-cooperative and occurs in the region from 0 to 60°C . Of course, we cannot exclude the possibility that the phase transition already sets in below 0°C . Consequently, a sharp T_m value cannot be defined. It is surprising that a *trans* double bond in the C_{18} chain gives rise to such strongly non-cooperative behaviour and that the plots of P vs. temperature for DEP and DOP vesicles are almost identical.

Interestingly, the difference in the behaviour induced by a *cis* and a *trans* double bond in the vesicular bilayer of the sodium di-*n*-alkyl phosphate vesicles is not as pronounced as in PC liposomes. Obviously, the reduction in the flexibility of alkyl chains has a more important influence on the phase transition than the introduction of a kink.

The most important difference between the gel and liquid-crystalline phase in bilayers is that above T_m the alkyl chains are more fluid than below T_m . In both phases, there is efficient packing of the alkyl chains in the bilayer. The introduction of a double bond does not significantly reduce the Van der Waals' contacts between the surfactant monomers in the bilayer, even if this leads to a kink in the extended alkyl chain as is the case for the *cis* isomer. *Cis*-unsaturated alkyl chains can be packed in a bilayer as efficiently as *trans*-unsaturated alkyl chains. But a double bond is a stiff segment in an alkyl chain, irrespective of its configuration, and it is apparently this decrease in flexibility that causes the non-cooperativity of the phase transition of the bilayer. Seelig and Seelig^{10b}

fluorescence polarization DOP and DEP vesicles

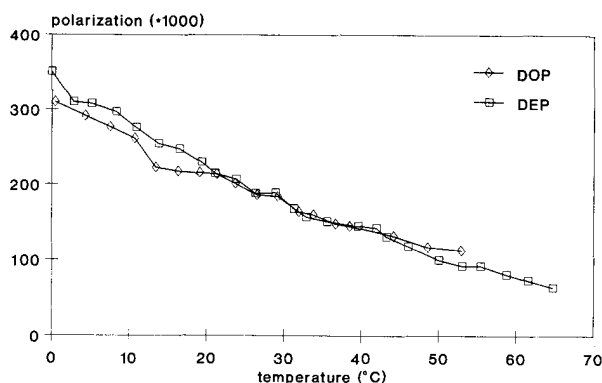


Figure 3. Plot of the fluorescence polarization (P) vs. T ($^{\circ}\text{C}$) for DEP and DOP.

proposed that the specific effect of a *cis* double bond in an alkyl chain of a lipid is caused by its unique configuration, rather than by its chemical nature. It is claimed that the presence of a *cis*-cyclopropane ring in an alkyl chain leads to comparable effects, but a *cis*-cyclopropane ring also reduces the flexibility of the alkyl chain. It is difficult to determine whether the non-cooperativity of the gel to liquid-crystalline phase transition of vesicles prepared from surfactants containing a *cis* double bond is due to an effect of the kink in the alkyl chains or to reduced flexibility.

The fluorescence polarization experiments did not show a difference between the phase transition behaviour of vesicles prepared from DOP or DEP, having a *cis* or a *trans* double bond in the middle of the alkyl chain. Unfortunately, it is not possible at the moment to assess possible effects due to different extents of water penetration into the bilayer of DOP and DEP. These effects may influence the viscosity and polarity of the specific binding sites of DPH in the bilayer, thereby affecting the polarization ratio. Nevertheless, we contend that the non-cooperativity of the phase transition from the gel to the liquid-crystalline state of DOP and DEP vesicles is primarily a result of the reduced flexibility of the alkyl chains.

³¹P NMR spectroscopy

Temperature-dependent ³¹P NMR spectroscopy is a sensitive tool for the analysis of the dynamic properties of the

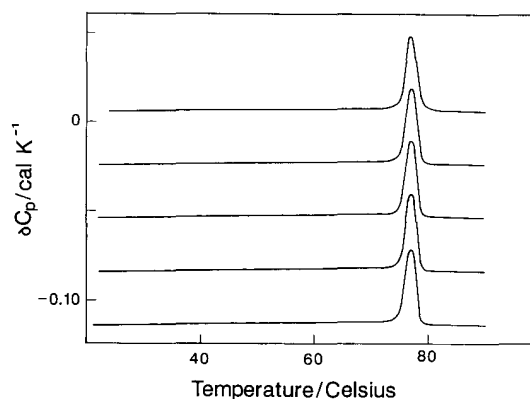


Figure 4. The effect of temperature on the differential heat capacity for aqueous solutions containing vesicles formed from DSP ($8.42 \cdot 10^{-3} \text{ M}$). The extremum at 77.1°C is recorded in each of several heat-cool-heat cycles recorded over a period of 16 h. The curves have been displaced for clarity on the heat capacity axis.

bilayer in vesicles prepared from synthetic surfactants with a phosphate headgroup. Both chemical shifts and line widths respond to a transition from the gel to the liquid-crystalline state.

DSP vesicles show a sharp increase of the line width and change in slope of the ^{31}P chemical shift vs. the temperature at 52°C. This temperature differs markedly from that identified by fluorescence polarization (72°C, *vide supra*). However, it is reasonable that these different techniques give different results. ^{31}P NMR detects effects due to the dynamics of the headgroup, in contrast to fluorescence polarization in which the mobility of a probe located in the interior of the bilayer is measured. The latter data provide more information about the fluidity of the alkyl chains.

As anticipated, DEP vesicles exhibited no dramatic changes of either the line width or the chemical shift in the ^{31}P NMR spectra as a function of temperature.

Differential scanning microcalorimetry (DSC)

DSC is a convenient technique for recording bilayer phase-transition temperatures T_m . For example, for sodium di-*n*-dodecyl phosphate, a single extremum is observed in the differential scan at 34.9°C. For vesicles formed from sodium di-*n*-tetradecyl phosphate, an extremum is recorded at 52.5°C. The scans are reversible and superimposable in terms of those recorded over a period of 12 hours following many heat-cool-heat cycles. We find that for DSP vesicles the phase transition occurs at 77.1°C (Figure 4), and is characterised by a bell-shaped change in heat capacity. It is clear that T_m is markedly dependent on the lengths of the alkyl chains in the di-*n*-alkyl phosphate. The fully reversible nature of the transitions provides good evidence that they are associated with physical rather than with chemical changes. The T_m determined with the fluorescence polarization (72°C) is in reasonable agreement with that determined by DSC. The slightly earlier apparent onset of melting in the fluorescence experiments may be determined by the precise location of the probe within the bilayer. Temperature-dependent ^{31}P NMR-spectroscopic data detect changes in the mobility of the surfactant headgroup. Using this technique, a phase transition is identified at 52°C. The contrast between the recorded scans for the DEP and DOP vesicles, having a *trans* or a *cis* double bond in the middle of the chain, is striking. In Figure 5 we report five scans recorded over a period of 10 hours for the DEP vesicles. Clearly, the phenomena responsible for the ex-

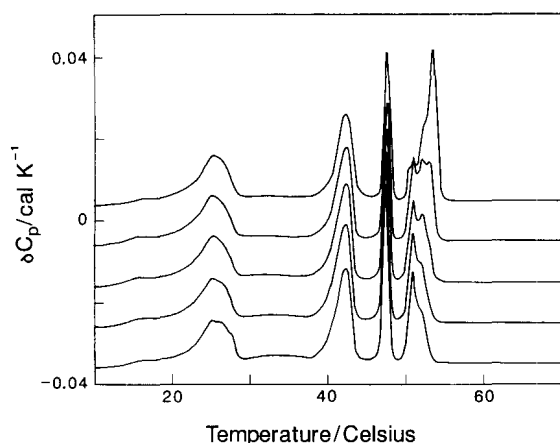


Figure 5. The effect of temperature on the differential heat capacity for aqueous solutions containing vesicles formed from DEP ($8.42 \cdot 10^{-3}$ M). The curves have been displaced for clarity on the heat capacity axis.

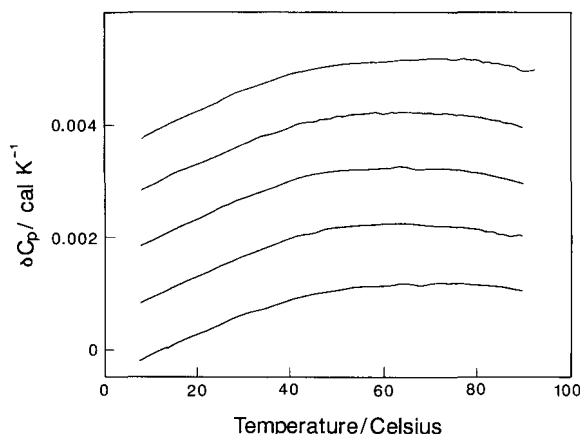


Figure 6. The effect of temperature on the differential heat capacity for aqueous solutions containing vesicles formed from DOP ($8.42 \cdot 10^{-3}$ M). The curves have been displaced for clarity on the heat capacity axis.

trema are reversible, at least for the main transitions at 25, 42, 47 and 53°C. As remarkable are the scans recorded for the *cis* derivative DOP, shown in Figure 6. Over the temperature range from 5 to 90°C, no extremum is observed. This is in agreement with the fluorescence polarization studies which also indicated a phase transition below 5°C.

Fusogenic properties of the vesicles formed from 1-3

Sodium di-*n*-alkyl phosphate vesicles fuse under the influence of the fusogenic agent calcium²⁵. The fusion of DOP vesicles was examined upon addition of Ca^{2+} ions (final concentration 4 mM) to a solution of the vesicles. The fusion process was monitored as a function of time by taking samples immediately and then after 15 and 30 minutes, 3½ and 17 hours (vesicles were stored at 60°C). Figures 7a-c show a series of electron micrographs of the DOP vesicles. Vesicle fusion is revealed by the vesicles becoming larger from the initial state, to immediately after calcium addition, to 15 or 30 minutes after calcium addition. When the vesicles were incubated for 20 hours, no further fusion took place. Overall, the vesicles become somewhat larger, but no giant vesicles are formed. Figure 8 graphically shows the size distribution of the different samples. The data for this diagram were obtained by first defining groups of vesicle sizes (e.g. 0-50 nm, 50-100 nm, etc.) and then counting the number of vesicles that fall into each group. The number of vesicles measured on each micrograph was always larger than 200. Another striking feature of this experiment was that calcium ions had no effect on the stability of the DOP vesicles. Solutions of DOP vesicles are stable for more than 1 week in the presence of 4 mM Ca^{2+} stored at room temperature or at 60°C. The DOP vesicles did not flocculate, nor could the formation of tubular structures be detected.

In the case of DDP and other di-*n*-alkyl phosphate vesicles, addition of calcium ions not only caused fusion, but also gave rise to the formation of long tubular structures, which have been identified as calcium di-*n*-alkyl phosphate crystals²⁶. In contrast to the usual behaviour of di-*n*-alkyl phosphate vesicles, DOP vesicles do not crystallize upon addition of calcium ions. Apparently, the formation of a crystal lattice is hampered by the presence of a *cis* double bond in the alkyl chains.

We anticipated that addition of EDTA would have no effect on the DOP vesicles. However, negative staining electron micrographs showed that large aggregates, vary-

ing in size from 400 to 1200 nm, had been formed (Figure 7d). Presumably, these aggregates are clusters of multilamellar vesicles (MLV). Freeze-fracture replicas of DOP vesicles were made of the vesicles (i) after 30 minutes incubation with Ca^{2+} (4 mM), and (ii) after addition of EDTA (10 mM). The first specimen showed fused vesicles identical in size to the fused vesicles observed in the negative staining experiments (100-400 nm). Figure 9a shows an example of these smoothly surfaced vesicles. Figure 9b shows the typical layered structure of the MLVs formed after the addition of EDTA.

This phase transition is reminiscent of phase transitions of phospholipids. *Papahadjopoulos et al.*²⁷ described the phase transition of phosphatidylserine (PS) liposomes. After the addition of Ca^{2+} to these vesicles, multilamellar structures were formed, many of which were cylindrically shaped. Addition of EDTA to these spiral aggregates produced large spherical, unilamellar vesicles. In our case, the effect of EDTA, as described above, occurs only when Ca^{2+} and EDTA are added in this sequence. When the fusogenic agent and the chelator were added in the reverse order (first EDTA, then Ca^{2+}), nothing happened.

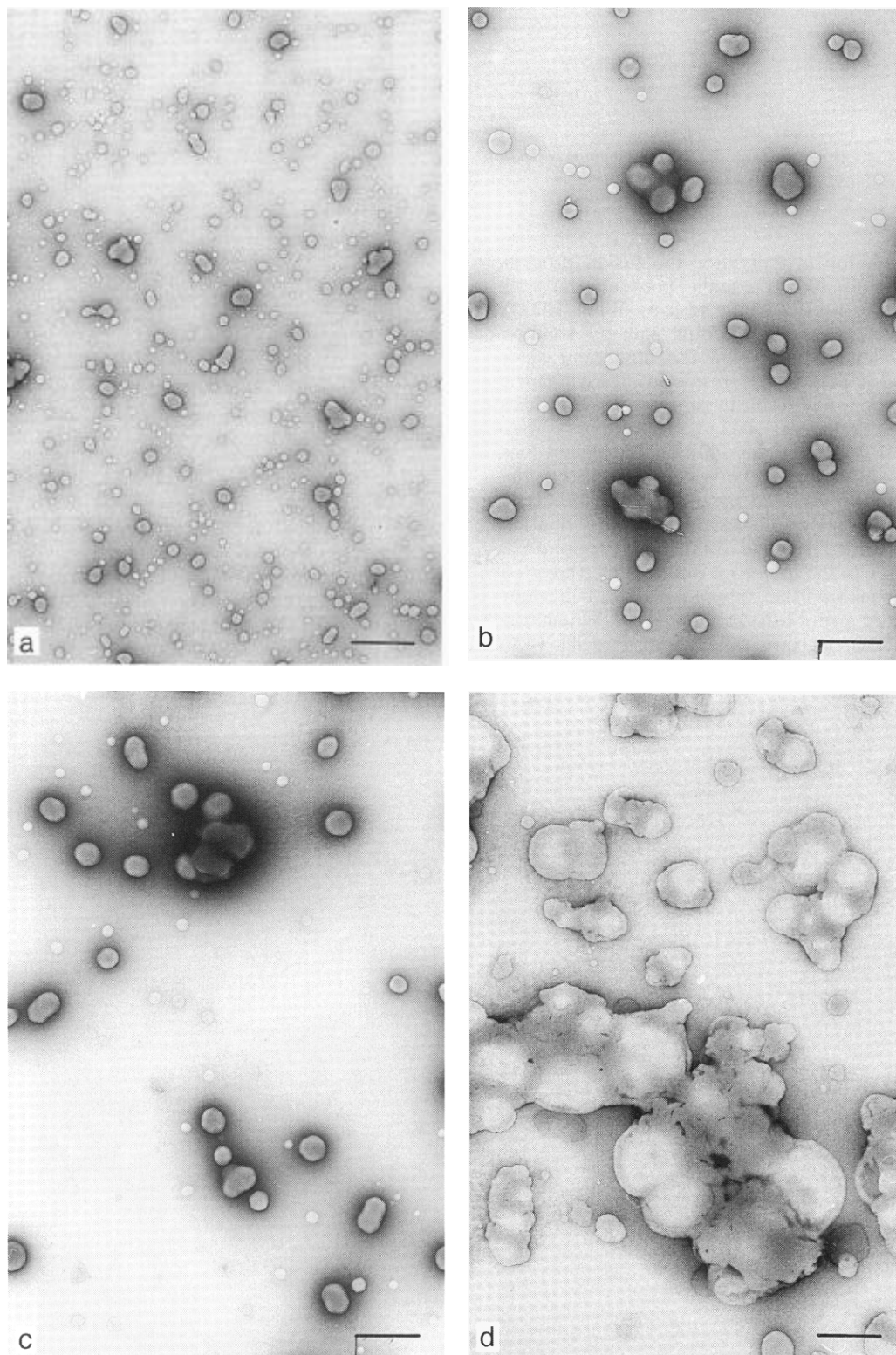


Figure 7. Electron micrographs of DOP vesicles (a) before Ca^{2+} addition, (b) vesicles incubated with Ca^{2+} for 1 min, (c) 30 min, and (d) after addition of EDTA. The bar represents 500 nm.

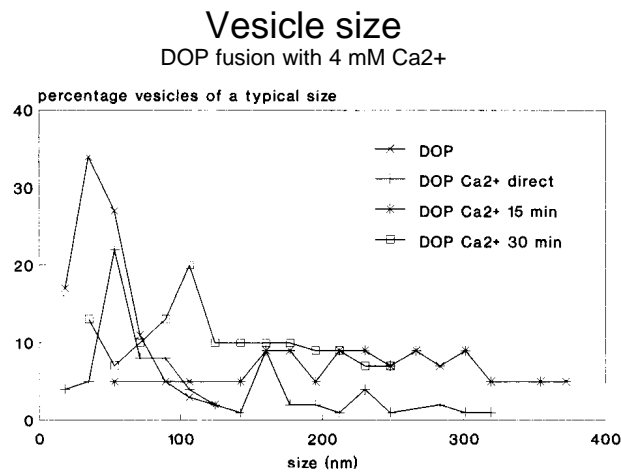


Figure 8. The effect of calcium ions on the size distribution of DOP vesicles. The number (%) of vesicles is plotted vs. size (nm).

A possible explanation is that after the fusion process, calcium ions are distributed equally between the bulk water phase and the interior of the vesicles. When EDTA is added, the calcium ions in the bulk aqueous solution are captured instantaneously. Now the inner and outer monolayer of the vesicle bilayer reside in an asymmetric media. This situation may result in rupture of the vesicles and the formation of MLVs.

In the case of DEP vesicles the addition of calcium ions resulted not only in fusion of the vesicles but also in the formation of crystals²⁶. An increase of the incubation time led to the disappearance of the vesicles and the formation of more crystals until DEP had completely precipitated. Addition of EDTA to the di-*n*-alkyl phosphates converted the calcium salt back into the sodium salt. This behaviour has been discussed previously by Fonteijn²⁸. When mechanical energy was put into this system, vesicles were

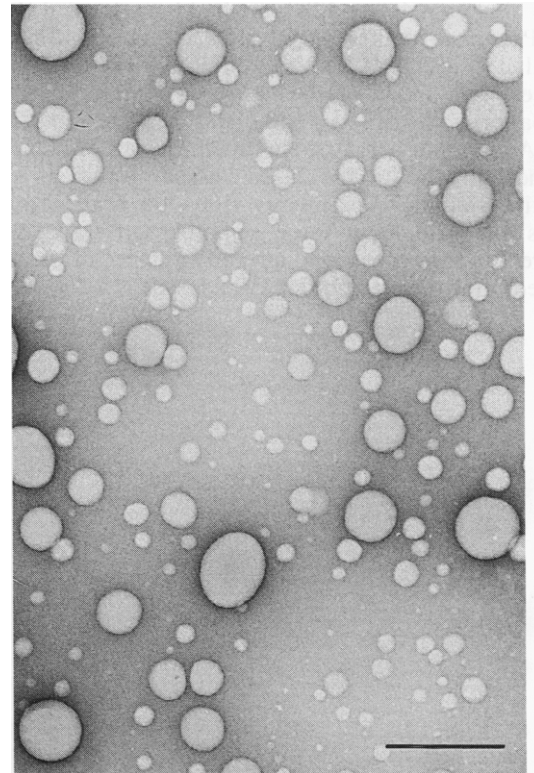


Figure 10. Electron micrograph of DEP vesicles after addition of EDTA. The bar represents 250 nm.

formed. Figure 10 shows micrographs of these rather small vesicles (50-200 nm).

Addition of calcium chloride to a solution of DSP vesicles resulted in the formation of crystals of the Ca²⁺ DSP salt. Addition of EDTA and subsequent input of mechanical energy caused the reformation of vesicles. EM micro-

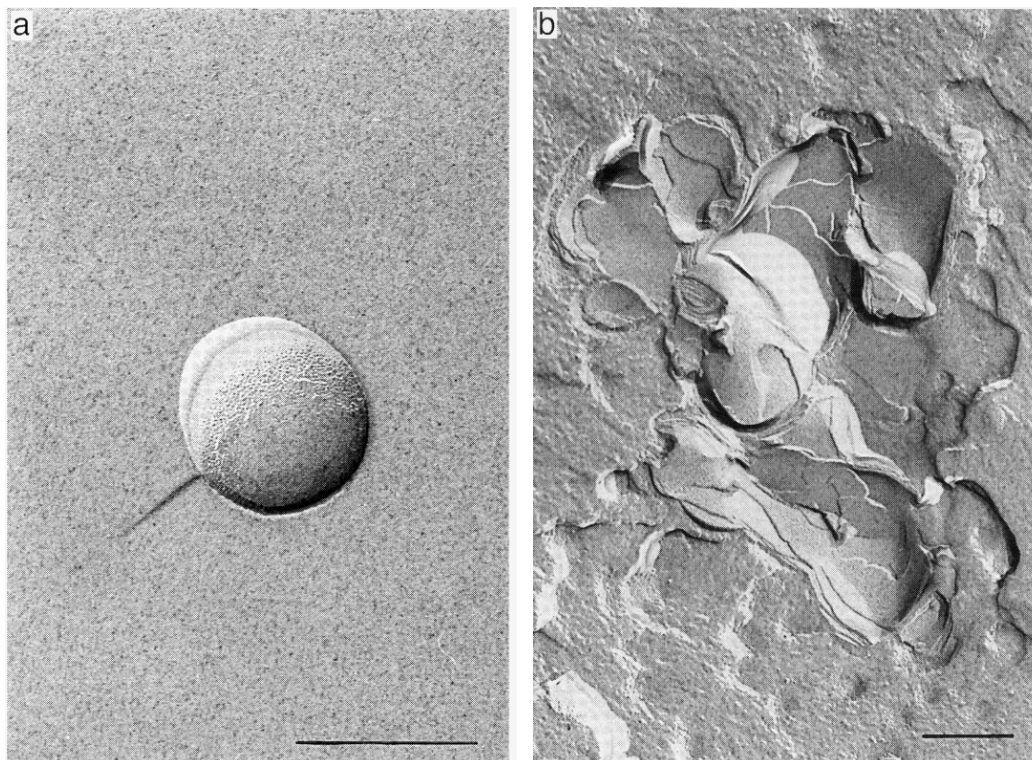


Figure 9. Freeze fracture EM of DOP vesicles (a) after 30 min. incubation with Ca²⁺ and (b) after addition of EDTA. The bar represents 250 nm.



Figure 11. Electron micrograph of DSP vesicles after subsequent addition of Ca^{2+} and EDTA. The bar represents 500 nm.

graphs show (Figure 11) multilamellar vesicles with a size of 100–1000 nm.

Conclusions

Electron-microscopy studies reveal that the three different di-*n*-alkyl phosphates DSP, DEP and DOP all form vesicles, despite the large differences in chain flexibilities. In fusion experiments, the three vesicle-forming surfactants behave differently. DEP and DSP vesicles flocculate when Ca^{2+} is added. After the addition of EDTA, vesicles are reformed. In contrast to all other di-*n*-alkyl phosphates studied to date²⁶, DOP fuses to give large vesicles when Ca^{2+} is added and does not crystallize. The fused vesicles are stable for a long time. However, when EDTA is added, a phase transition to multi-lamellar vesicles takes place.

The various vesicles show distinct thermal properties associated with the gel to liquid-crystalline phase transition arising from the different configurations of the hydrocarbon chains. The minor differences between the temperature effects of the fluorescence polarization of the *trans* and *cis* isomers indicates that the reduction of the flexibility of the alkyl chains has a dominant influence on the transition from the gel to liquid-crystalline phase. We contend that the introduction of a stiff segment probably causes a larger effect on the phase transition of the bilayer than the introduction of a kink.

Acknowledgements

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